Both mature KIR+ and immature KIR- NK cells control pediatric acute B cell precursor leukemia in NOD.Cg-Prkdcscid IL2rgtmWjl/Sz mice

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Abstract: Therapeutic natural killer (NK) cell-mediated alloreactivity towards acute myeloid leukemia (AML) has largely been attributed to mismatches between killer immunoglobulin-like receptors (KIRs) on NK cells and their ligands, HLA class I molecules, on target cells. While adult acute B cell precursor leukemia (BCP-ALL) appears to be resistant to NK cell-mediated lysis, recent data indicate that pediatric BCP-ALL might yet be a target of NK cells. We here demonstrate in a donor-patient-specific NOD.Cg-Prkdc(scid) IL2rg(tmWjl)/Sz (NSG) xenotransplantation model that NK cells mediate considerable alloreactivity towards pediatric BCP-ALL in vivo. Notably, not only adoptively transferred mature, KIR(+) NK cells but also immature, KIR- NK cells arising early post transplantation in humanized NSG mice (huNSG) exerted substantial anti-leukemic activity. Low-dose and long-term treatment of huNSG mice with the DNA-demethylating agent 5-Aza-cytidine distinctly enhanced the anti-tumor response, interestingly without inducing common inhibitory KIR expression but rather by promoting the differentiation of various NK cell precursor subsets. Collectively, these data indicate that the future design of innovative therapy protocols should consider further exploitation of NK cell-mediated immune-responses for poor prognosis pediatric BCP-ALL patients.

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Running Title: NK cells control pediatric BCP-ALL *in vivo*.

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KEY POINTS

1. Both mature KIR^+ and immature KIR^- NK cells exert anti-leukemic activity towards pediatric BCP-ALL *in vivo*.
2. *In vivo* treatment with low-dose 5-Aza-cytidine enhances immature and mature NK cell counts and promotes anti-tumor response.

ABBREVIATIONS

BCP-ALL	acute B cell precursor leukemia
DNMT	DNA methyltransferase
GvL	Graft versus leukemia
huNSG	humanized NOD.Cg-*Prkdc*^{scid} *IL2rg*^{tmWjl}/Sz mice
HSC	hematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
KIR / KIRL	killer immunoglobulin-like receptor / KIR ligand
NKAES	NK cell activation and expansion system

ABSTRACT

Therapeutic natural killer (NK) cell-mediated alloreactivity towards acute myeloid leukemia (AML) has largely been attributed to mismatches between killer immunoglobulin-like receptors (KIRs) on NK cells and their ligands, HLA class I molecules, on target cells. While adult acute B cell precursor leukemia (BCP-ALL) appears to be resistant to NK cell-mediated lysis, recent data indicate that pediatric BCP-ALL might yet be a target of NK cells. We here demonstrate in a donor-patient-specific NOD.Cg-*Prkdc*^{scid} *IL2rg*^{tmWjl}/Sz (NSG) xenotransplantation model that NK cells mediate considerable alloreactivity towards pediatric BCP-ALL *in vivo*. Notably, not only adoptively transferred mature, KIR^{+} NK cells but also immature, KIR^{-} NK cells arising early post transplantation in humanized NSG mice (huNSG) exerted substantial anti-leukemic activity. Low-dose and long-term treatment of huNSG mice with the DNA-demethylating agent 5-Aza-cytidine distinctly enhanced the anti-tumor response, interestingly without inducing common inhibitory KIR expression but rather by promoting the differentiation of various NK cell precursor subsets. Collectively, these data indicate that the future design of innovative therapy protocols should consider further exploitation of NK cell-mediated immune-responses for poor prognosis pediatric BCP-ALL patients.

INTRODUCTION

Natural killer (NK) cells belong to innate lymphoid immune cells that contribute to anti-tumor responses without prior sensitization. One important receptor family that controls NK cell activity constitutes the HLA class I-restricted killer immunoglobulin-like receptor (KIR) family that comprises both inhibitory and activating family members which recognize allotypic variants of HLA class I alleles as KIR ligands (KIRL). In the setting of haplo-identical hematopoetic stem cell transplantation (HSCT), NK cells maturing in a HLA-disparate recipient will be shaped in the predominantly donor-type-like hematopoietic niche and will thus be donor-tolerant and recipient-"alloreactive", at least during the first months after reconstitution.[1-3] In this regard, the beneficial effects of promoting a certain degree of donor-recipient HLA-disparity was initially described by the Perugia group that provided evidence that allogeneic HSCTs performed with grafts from KIR-KIRL mismatched donors promoted NK cell alloreactivity and enhanced survival rates in adult patients with acute myeloid leukemia (AML) but not with B cell precursor leukemia (BCP-ALL).[4,5] As a result of this apparent resistance of adult BCP-ALL to NK cell-mediated lysis, the usefulness of NK cell immune-responses in treating BCP-ALL has been questioned and research in this field has largely been neglected. However, recent data indicate that the disease entity of pediatric BCP-ALL might yet be a target of "alloreactive" NK cells.[1,2,6-8] Considering the various potential facets of NK cell immune therapy such as adoptive transfer of mature NK cells, co-transfer of mature NK cells during graft manipulation, emergence of immature NK cells early post transplantation and the currently existing limited number of clinical studies in BCP-ALL-bearing children, we sought to define conditions under which NK cell-mediated alloreactivity towards pediatric BCP-ALL could be fully exploited.

MATERIALS AND METHODS

Mice

NOD.Cg-*Prkdc*^{scid} *IL2rg*^{tmWjl}/Sz (NSG) mice were purchased at "The Jackson Laboratory" and maintained under specified pathogen-free conditions in the research animal facility of the University of Tuebingen, Germany. All experimental animal procedures were conducted according to German federal and state regulations.

Induction of leukemia in NSG mice

Patient-specific leukemia was induced in NSG mice as described before.[9] The study was approved by the local ethics committee and written informed consent was obtained from the parents, in accordance with the Declaration of Helsinki. Upon engraftment, mice were sacrificed and bone marrow (BM) or spleen specimens of leukemia-bearing mice were stored at -80° C for *de novo* generation of patient-specific leukemia at later time points.

NK cell activation and expansion system (NKAES)

Peripheral blood mononuclear cells (PBMCs) from HLA-typed donors (sequence-based typing with 4-digit resolution) (Supplemental Table S1) were used with informed consent to expand NK cells in a medium containing 100 IU/ml IL2 (Novartis, Switzerland) using irradiated, membrane bound (mb)IL15-41BBL expressing K562 cells generously provided by D. Campana (University Children's Hospital, Singapore) at a ratio of 1:1.5.[10] To expand NK cells in a GMP-guided fashion, pooled human fresh frozen plasma instead of FCS was used. Expanded cells were harvested after 14 days and frozen in the presence of 10% DMSO at -80° C. Prior to experiments, NKAES cells were thawed and cultured overnight in the presence of 100 IU/ml IL2 for *in vitro* cytotoxicity assays or adoptive transfer experiments

and 200 IU/ml IL2 for functional NK cell response staining or alloreactive NK cell subset analysis.

Adoptive NK cell transfer

Donor-patient-pairs were selected that provided a mismatch or match in their KIR-KIRL repertoire (Table 1; Supplemental Tables S1 and S2). 1 x 10^6 blasts were intravenously injected into unirradiated NSG mice on d0, followed by intravenous injection of 10 x 10^6 NKAES cells at the indicated time points. To monitor engraftment of leukemia, PBMCs of NSG mice were subjected to flow-cytometric analysis using formerly defined patient-specific leukemia surface markers.

Humanization of NSG mice

Humanization of NSG mice was performed as described before.[11] Following approval by the local ethics committee, parents donated < 5% of the hematopoietic stem cells (HSCs) for humanization of NSG mice. In selected experiments, HLA-typed HSCs were purchased from Key Biologics (Memphis, TN, USA). To induce maturation of NK cells, huNSG mice were treated at 12 weeks post transplantation with a complex of IL15/IL15Rα.[12] In addition, huNSG mice were injected once on d15 post initiation of IL15 stimulation by intraperitoneal administration of 100 µg poly I:C (Sigma Aldrich, Taufkirchen, Germany).[13]

Determination of *in vivo* cytotoxicity

Individual donor-patient-pairs were selected that provided a mismatch or a match with respect to their KIR-KIRL repertoire. Mice were transplanted with HSCs of the respective donor and activated as described above at 12-20 weeks post transplantation. To study NK cell-mediated cytotoxicity, 3 x 10^6 blasts were intravenously injected into huNSG and the extent of blasts was determined at 20hrs post injection in the BM using polychromatic (8-11)

color flow-cytometry. In some experiments, a mixture of 3 x 10^6 NSG-derived blasts (total 6 x 10^6 blasts) of two patients with varying KIR-KIRL repertoires were injected into huNSG mice and leukemia was quantified using the hierarchical gating strategy depicted in Figure S5A. In all experiments, the frequencies of patient-specific vital blasts were normalized to vital murine $CD45^+$ cells.

In vivo treatment of huNSG mice with 5-Aza-cytidine

On d39 post transplantation, huNSG mice were randomly assigned to treatment or control group and therapy with 5-Aza-cytidine (0.025mg/mouse/dose intraperitoneally, twice a week for a total of 4 weeks) or PBS as control was initiated. 4 weeks later, 3 x 10^6 blasts were intravenously injected into 5-Aza-cytidine or sham-treated animals. 20hrs later, mice were sacrificed and subjected to analysis of NK cell phenotype and for quantification of leukemic burden.

Functional NK cell response staining

Sorted KIR^+ and KIR^- (for the respective sorting KIR mAb cocktail refer to Suppl. Data) NKAES subsets were co-cultured for 6hrs with NALM-16 or K562 cells (E:T ratio of 1:2) in the presence of CD107a-APC (H4A3) (BD Pharmingen). Subsequently, NK cells were stained with the indicated surface antibodies, permeabilized and co-stained with the respective intracellular antibodies (Perforin-PB (dG9) and TNF-bv605 (MAb11)) (Biolegend). For determination of IFN-γ, unsorted NKAES cells were co-cultured with the respective target cells, were subsequently stained with surface antibodies (including the above mentioned anti-KIR antibody cocktail), and finally stained with IFN-γ-BUV395 (B27) antibody (BD Horizon). Percentages of the respective NK cell subpopulation were then normalized to the baseline levels of NKAES cells cultured in control medium only. The specificity of the intracellular perforin staining was additionally verified by determining the perforin

concentration in the co-culture supernatants using the Diaclone Perforin ELISA KIT (ACTIVE BIOSCIENCE, Germany).

Alloreactive NK cell subset analysis

Unsorted NKAES cells were co-cultured with the respective target cells in the presence of CD107a-bv421 (H4A3) and subsequently stained with the following mAbs: KIR3DL1 (DX9, BD Pharmingen), KIR2DL2/L3/S2 (GL183, Beckman Coulter), KIR3DL1/S1 (Z27.3.7, Beckman Coulter) and KIR2DL1/S1/S4 (HP-3E4, BD Pharmingen). The selective combination of anti-KIR mAbs with different or identical fluorochromes hereby allowed the discrimination KIR^- and non-alloreactive or alloreactive KIR^+ NK cell subsets in the context of Bw4/C1 target cell recognition.

Statistics

Statistic advice was obtained from M. Eichner, PhD, Department of Clinical Epidemiology and Applied Biometry, University of Tuebingen, Germany, and M. Urschitz, MD, Institute of Medical Statistics, University of Mainz, Germany. Mean values and SEM from experiments with 2 conditions were analyzed with the Student *t* test. The effect size Θ was calculated using the standardized mean difference between two populations: $\Theta = (\mu 1 - \mu 2) / \sigma$, where μ1 and 2 represent the mean values of the two study groups and σ represents the SEM of total study population.

Methods concerning flow-cytometric analyses, determination of *in vitro* cytotoxicity and KIR-Q-PCR are referred to in Supplemental Materials and Methods.

RESULTS

KIR-KIRL mismatch constellations promote the alloreactivity of cytokine-matured NK cells towards pediatric BCP-ALL *in vitro* and *in vivo*.

To characterize the alloreactivity of mature NK cells in various KIR-KIRL repertoire constellations, we applied a previously described good manufacturing process (GMP)-guided NK cell activation and expansion system (NKAES)[10] and generated large numbers of cytokine-matured NK cells of donors with defined HLA class I genotypes (Supplemental Table S1), KIR repertoires (Supplemental Table S2) and NK cell surface phenotypes (Supplemental Figure S1A, B). We then chose a pediatric AML (Kasumi-1) and a BCP-ALL cell line (NALM-16) which express important NK cell receptor ligands to a comparable and significant extent (Figure S1C) to test whether KIR-KIRL mismatched NK cells can in principle target pediatric BCP-ALL. Indeed, KIR-KIRL mismatched NK cells exerted alloreactivity not only towards a pediatric AML but also a BCP-ALL cell line (Figure S2A). Comparative analysis of *in vitro* cytotoxicity assays performed with other pediatric BCP-ALL cell lines hereby demonstrated that this alloreactivity was clearly dependent on the extent of NKG2DL expression (Figure S2B, C). To further describe the relevance of KIR-KIRL interactions that might provide functional NK cell competence in addition to the immanent NKG2D-restricted activation, we performed cytotoxicity assays with donors that exhibited either a KIR-KIRL mismatch or a match towards two given BCP-ALL specimen (P3B relapse, P31G) (Table 1). *In vitro* cytotoxicity assays demonstrated that alloreactivity to BCP-ALL was better for those NK cells that were not subject to inhibition by self-HLA molecules (Figure 1A). In addition, injection of NKAES cells from a KIR-KIRL mismatched donor into P3B-engrafted NSG mice resulted in a higher reduction of tumor burden than transfer of the respective control NK cells (Figure 1B, C).

The KIR$^+$ NK cell subset of KIR-KIRL mismatched donors exerts higher cytotoxicity towards BCP-ALL than the corresponding KIR$^-$ subset.

To further characterize the functional repertoire of NKAES cells with regard to potential KIR-KIRL mismatch constellations, we sorted KIR$^+$ and KIR$^-$ NK cell subsets. The KIR$^+$ NK cell subset of KIR-KIRL mismatched donors uniformly demonstrated higher levels of cytotoxicity when compared to the respective KIR$^-$ controls (Figure 2A), a phenomenon that became even more evident upon normalization (Figure 2B). In contrast, the KIR$^+$ NK cells of matched donors invariably exhibited impaired cytotoxicity, indicating that functionality might have been compromised in response to inhibitory KIR-KIRL interactions. Cytotoxicity assays performed in the presence of the common inhibitory KIR2DL1 and 2DL2/3 blocking mAb IPH2102[14] revealed that the lytic activity of KIR$^+$ NK cells of KIR-KIRL matched, HLA-C1 homozygous donors must have been controlled by inhibitory interactions of KIR2DL2 and 2DL3 receptors with their respective HLA-C1 group ligands expressed on NALM-16 cells (Figure 2C). In contrast, the KIR2DL2 and 2DL3$^+$ NK cell subset of our KIR-KIRL mismatched, HLA-C2 homozygous donors did not respond to inhibitory KIR blockade.

The alloreactive KIR$^+$ NK cell subset of KIR-KIRL mismatched donors exhibits a superior ability for degranulation in response to pediatric BCP-ALL.

Facing this high cytotoxic activity of KIR$^+$ NK cells towards KIRL-negative BCP-ALL target cells, one would expect significant differences in assays quantifying the ability for cytokine secretion or degranulation. Indeed, KIR-KIRL mismatch constellations boosted to some extent the ability of KIR$^+$ NK cells for both (Figure 3 and S3), however, these data did not reach statistical significance. To further dissect the extent of anti-tumor responses of KIR$^+$ NK cells, we therefore analyzed the ability for degranulation in the alloreactive and non-alloreactive NK cell subsets (Figure 4A, for the respective NK cell phenotype see Figure S1B). Alloreactive KIR$^+$ NK cells from mismatched donors revealed a superior capacity for degranulation in comparison to alloreactive KIR$^+$ NK cells from matched donors (Figure 4B-

D). This was first of all true for the KIR^+NKG2A^- NK cell subset but to some extent also for the cytokine-activated and thus expanded KIR^+NKG2A^+ subset. Interestingly, the KIR-KIRL mismatched donor SNK14B had very little alloreactive KIR^+ NK cells (Figure S4) explaining the comparatively poor killing ability depicted in Figure 2A. In summary, we at this point conclude that cytokine-matured NK cells of KIR-KIRL mismatched donors can exert a substantial cytotoxicity towards pediatric BCP-ALL cells which is above all conferred by vivid degranulation of alloreactive KIR^+ NK cells.

Immature, KIR^- NK cells exert alloreactivity towards pediatric BCP-ALL.

Since humans first reconstitute a large pool of immature KIR^- NK cells early post transplantation[2,15,16], we next asked whether these NK cells might also exert effector functions against pediatric BCP-ALL. As KIR acquisition in NSG mice transplanted with human hematopoietic stem cells (HSC) (huNSG) will occur only in a minor fraction of huNSG-derived NK cells (André MC & Münz C, own unpublished data), we chose this NSG xenotransplantation model as a surrogate model for the generation of "pseudo-mature lytic NK cells"[17] arising in the early post transplantation period in humans (Figure 5A). The injection of a primary BCP-ALL specimen (P23T) into huNSG mice pre-stimulated with IL15/IL15Rα and poly I:C resulted in a substantial reduction of P23T tumor burden compared to control mice (Figure 5B). Since these results suggested that huNSG-derived NK cells may in principle be functionally active, we next injected a pooled sample of equal numbers of two primary BCP-ALL samples (P3B and P23T) with different KIRL repertoires into huNSG mice (Figure 5C). Applying the sequential gating strategy depicted in Figure S5A and verifying that the huNSG-derived NK cells were indeed to a large extent negative for common KIRs (Figure 5D), we found that the KIR-KIRL mismatched sample P3B was significantly better killed than the matched sample P23T (Figure 5E). Of note, we deliberately euthanized huNSG mice 20hrs post injection of leukemia to exclude for T cell-mediated responses as a major cause of leukemia reduction. As the alloreactivity towards P3B and P23T was reversible in

experiments performed with a different HSC donor (Figure S5B), we conclude that the alloreactivity towards P3B depicted in Figure 5E was not a result of a P3B-intrinsic slower growth rate. Given that HLA class I, ICAM-1, NKG2D and DNAM-1 ligands (Figure S1C) were expressed to a comparable extent by P3B and P23T, we assume that either KIR-KIRL interactions other than the one analyzed by us (KIR2DL1/S1/S4, KIR2DL2/S2/L3 and 3DL1) or modest differences, i.e. in the expression of NKG2DL, must have contributed to this phenomenon.

"Bridging" therapy with 5-Aza-cytidine supports NK cell alloreactivity towards pediatric BCP-ALL in the early post transplantation period.

To elaborate the full potential of the immature NK cell pool arising early post-transplantation, we recapitulated existing *in vitro* data indicating that 5-Aza-cytidine modulates NK cell function by inducing KIR promoter activity[18] and expression[19] in our xenotransplantation model in the context of pediatric BCP-ALL disease. As the therapeutic efficacy of DNA methyltransferase (DNMT) inhibitors may reach from the induction of DNA-demethylating effects at low doses to the induction of direct cytotoxic effects at higher doses, we decided to apply a low-dose treatment regimen for an extended period to potentially enable long-term epigenetic modulation of NK cells (Figure 6A). This treatment regimen resulted in a statistically not significant reduction of both human and murine $CD45^+$ cells, reflecting to some extent a drug-induced BM cytotoxicity (Figure 6B, C). Interestingly, contrary to previously published *in vitro* data reporting a 5-Aza-cytidine induced functional inhibition of mature NK cells[20], we observed a clearly reduced BCP-ALL burden in 5-Aza-cytidine-treated huNSG mice (Figure 6D, F). Of note, we had stopped 5-Aza-cytidine therapy three days before the injection of leukemia and the half-life of the drug is described to be less than 4hrs.[21] Together with our data obtained in non-humanized controls (Figure 6E), we can therefore largely exclude a drug-induced, direct cytotoxic effect that might have reduced the tumor load.

Low-dose and long-term exposure to 5-Aza-cytidine promotes NK cell ontogeny.

NK cell receptor analysis of huNSG mice revealed that 5-Aza-cytidine did not significantly modulate the expression of selected NK cell receptors (KIRs, NKG2A, NKp44 and NKG2D) at the low doses applied (Figure 7A, B). However, the analysis of NK cell subsets interestingly showed that 5-Aza-cytidine-treated huNSG mice had clearly higher numbers of both immature ($CD34^+CD117^+$ and $CD34^-CD117^{low}CD94^-$) NK cell precursors and mature ($CD34^-CD117^-CD94^+NKp46^+$ and $CD34^-CD117^-CD94^-NKp46^+$) NK cell subsets[22] (Figure 7C), indicating that 5-Aza-cytidine might have facilitated NK cell ontogeny itself.

DISCUSSION

Given the disparity of adult and pediatric acute lymphoid leukemia, we here sought to investigate NK cell-based immune responses to the tumor entity pediatric BCP-ALL. Our data reveal that not only mature KIR^+ but interestingly also immature KIR^- NK cells control pediatric BCP-ALL *in vivo* and indicate that future protocols should consider further exploitation of NK cell-mediated immune-responses for poor prognosis pediatric BCP-ALL patients.

The difference in the susceptibility of adult and childhood BCP-ALL to NK cell-mediated lysis has been mainly ascribed to differing expression of cell adhesion molecules of the β1 and β2 integrin and the Ig superfamily, respectively, that essentially results in a reduced NK cell-target conjugate formation and activation in the case of adult BCP-ALL.[4,23] In addition, the surface density of HLA class I ligands is greater and the expression of ligands to major activating NK cell receptors such as NKG2D is lower in pediatric BCP-ALL as compared to AML.[24] In line with this, our group earlier demonstrated that the *in vitro* cytolytic activity of NK cells against BCP-ALL in part correlated with the extent of MHC expression on the respective specimen.[6,7]

Accounting for our previous work that demonstrated that the injection of patient-specific leukemia into NSG mice results in the constitution of a model that reflects individual leukemogenecity[9], we analyzed the leukemic burden in BCP-ALL-bearing mice upon adoptive NK cell transfer and demonstrated that KIR-KIRL mismatched NK cells targeted but did not eliminate pediatric BCP-ALL. Mechanistically, the interaction of NK cells with BCP-ALL was accompanied by a heightened functionality of alloreactive KIR^+ NK cells of KIR-KIRL mismatched donors, particularly in terms of their ability for degranulation. As the presence of the inhibitory KIR blocking mAb IPH2102 distinctly enhanced the cytotoxicity of matched KIR^+ NK cells, we conclude that the KIR-KIRL axis indeed contributes to the control of NK cell alloreactivity and hypothesize that this antibody might provide clinically significant

effects in selected young BCP-ALL patients. The absence of effectiveness of KIR blockade in our KIR-KIRL mismatched C2/C2 donors is difficult to explain as inhibitory signaling alone should be functional even in KIR2DL2/L3$^+$ NK cells of these donors. However, the KIR2DL2/L3$^+$ NK cell subset that lacked the co-expression of other inhibitory KIRs was comparatively large in our donors and displayed, despite the presence of NKG2A, a low functional ability in terms of response to K562 (data not shown). Thus, we at this point assume that these two findings together might explain the decreased responsiveness to IPH in our C2/C2 mismatched donors.

Irrespective of the individual KIR-KIRL constellation, the KIR$^-$ NK cell subset interestingly enough displayed substantial functionality. In line with the previously published observation that the attenuated function of KIR$^-$ NK cells can be overcome by exposure to cytokines[25], we attribute this observation to the cytokine-rich expansion protocol.

The so far published data on adoptive NK cell transfer in children is restricted to feasibility studies performed mostly in AML patients.[26] Our group recently demonstrated that the infusion of IL15-stimulated CD3$^-$/CD19$^-$-depleted HSC grafts (containing high numbers of NK cells) is safe even in the haplo-identical setting.[8] Given that NK cells can persist and expand in HLA-mismatched hosts[27] and that the transfer of mature NK cells does obviously not promote graft-versus-host-disease (GvHD)[8,26], the data depicted here support the concept that the adoptive transfer of KIR$^+$ NK cells of KIR-KIRL mismatched donors might indeed play a role in the treatment of relapsing BCP-ALL disease, not alone but as an adjunct to haplo-identical HSCT.

To date, two clinical studies evaluated the feasibility of KIR-KIRL mismatched haplo-identical HSCTs in children with BCP-ALL. Our group demonstrated that the graft-versus-leukemia (GvL) effect best correlated with the number of KIR-KIRL mismatches.[1] The second study was performed by the Moretta group with the aim of better defining the specificity and clonal distribution of “alloreactive” NK cells.[2] Collectively, these two preliminary studies show that the incorporation of theoretical assumptions on KIR-KIRL interactions in haplo-identical

HSCTs of children with BCP-ALL is feasible, that such a mode of donor selection does not heighten the risk of GvHD and that the analysis of receptor-ligand constellations may have prognostic implications.

We here systematically demonstrate in huNSG mice that emerging KIR$^-$ NK cells exert a considerable cytotoxicity towards BCP-ALL, thus extending earlier data that a substantial number of phenotypically healthy appearing NK cells exist in mice and men that obviously lack the expression of any inhibitory receptor for "self"-HLA class I[28,29] but nevertheless display a certain degree of functionality. In line with previously published data that "unlicensed" NK cells may exert a profound immune response in the context of murine cytomegalovirus infection[30], our data indicate that the anti-tumor properties of immature KIR$^-$ NK cells are substantial and might just as well have been previously underestimated. As the KIR expression in huNSG mice was despite the administration of IL15/IL15Rα complex negligible, we at this point do not ascribe the occurring NK cell activation to the lack of KIR-KIRL-mediated inhibition but rather assume that receptor-ligand interactions other than the one analyzed by us must have contributed to this phenomenon.

Using a humanized xenotransplantation model to analyze GvL potency after haplo-identical HSCT is undoubtedly technically challenging and has to our knowledge so far not been attempted. The application of minimal residual disease (MRD) analysis based on multi (8-11) parameter flow-cytometry enabled the distinction of immunophenotypic features of one (or two) patient-specific malignantly transformed B cell specimen from early-arising donor-type like B cell-lineage precursors. This approach has allowed us to characterize GvL effects in a dynamic and largely functional hematopoietic system that incorporates complex effector-target cell interactions in the presence of "human-like" bystander cells and supportive cytokines.

As recent data indicate that DNA demethylating agents may not only affect the growth of malignant cells but might also modulate inhibitory KIR expression on NK cells, we tested 5-Aza-cytidine in our huNSG model. Interestingly, we observed a significantly reduced BCP-

ALL burden in huNSG mice that could not be solely attributed to drug-induced, direct cytotoxic effects. This observation is in contrast to previously published *in vitro* data reporting a 5-Aza-cytidine-induced functional inhibition of NK cells[20,31], however, careful analysis of these data reveals that the knowledge of 5-Aza-cytidine mediated immune-modulation of NK cells is basically restricted to studies of mature NK cells of healthy volunteer donors. Analysis of BM-residing NK cell subsets in huNSG mice demonstrated that 5-Aza-cytidine-treated mice harbored distinctly higher numbers of both immature and mature NK cell subsets. So far, it has been speculated that beneficial anti-tumor effects of 5-Aza-cytidine should probably be attributed to the induction of HLA class I expression and cancer testis antigens on malignant or pre-malignant cell clones.[32] In line with this tumor-focussed concept, 5-Aza-cytidine is used to "bridge" the early post transplantation period in high risk AML and chronic myelomonocytic leukemia (CMML) patients.[33,34] However, based on our results, we propose that 5-Aza-cytidine therapy might induce a re-modelling in developing NK cell precursors that – presumably together with an altered DC functionality and a fine-tuned NK cell-promoting cytokine milieu[35] – leads to increased NK cell numbers and improved functionality.

In line with the observation that 5-Aza-cytidine may promote differentiation of malignantly transformed cells by inducing the re-expression of epigenetically down-regulated PU.1[36] and considering the crucial role that PU.1 plays in the regulation of NK cell differentiation and homeostasis[37], we assume that the re-expression of PU.1 might have promoted NK cell differentiation in our model. In addition, it has been shown for low but not high dose 5-Aza-cytidine therapy that this may increase the number of cells in the S phase and may thus promote cell cycle progression.[38] Recalling data that 5-Aza-cytidine therapy does not evoke a broad and unspecific passive demethylation of the genome but rather induces a highly specific non-random demethylation pattern[38], we at this point hypothesize that low dose 5-Aza-cytidine might have selectively re-shaped factors important for NK cell transcription and cell cycle control.

In summary, we here provide substantial evidence that both KIR^+ but also immature KIR^- NK cells may exert clinically relevant GvL effects towards pediatric BCP-ALL. Our data thus

support the rationale for adoptive NK cell transfer of *ex vivo* activated mature donor-specific NK cells that would chaperone the early, NK cell-deficient phase of ideally KIR-KIRL mismatched haplo-identical HSCTs in children with relapsing BCP-ALL (Figure 8). The application of a low dose 5-Aza-cytidine “bridging” therapy - potentially under supportive cytokine administration - might additionally promote NK cell differentiation and functionality and would thus constitute an optional treatment strategy for those patients lacking a suitable KIR-KIRL mismatched donor.

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AUTHORSHIP CONTRIBUTIONS

AK and JW designed and performed experiments, interpreted the data and critically revised the manuscript, KEW designed the patient-specific MRD analysis, contributed to performing and interpreting the MRD data obtained in mice, KEW and HJB advised and helped with cell sorting, ME provided patient's samples, LO and MM established the KIR-Q-PCR, PL obtained written informed consent of parents and patients, WH and RH interpreted the data, UFH and CM interpreted the data and critically revised the manuscript, MCA conceptualized the work, designed experiments, interpreted data and wrote the paper.

DISCLOSURE

MCA and AK have filed a provisionary patent application on the use of IPH2102 in pediatric BCP-ALL. None of the other authors declare a conflict of interest.

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TABLE 1. KIR-KIRL repertoire constellations of donors and patients included in this study. Given is the internal de-identification code of the NK cell donors (SNK), stem cell donors (SSC), the donor-specific inhibitory KIR repertoire relevant for characterization of the alloreactive subset, the KIRL repertoire of the respective BCP-ALL and the identification of the educated KIRs that are specific for the missing KIRL in the target cells. Note, that KIR2DL1 and KIR2DL2/3 KIR family members recognize HLA-C alleles with Lys^{80} (C2 epitope) or Asn^{80} (C1 epitope) residues, respectively, whereas KIR3DL1 recognizes HLA-A and B alleles with a Bw4 supertypic specificity and KIR3DL2 recognizes HLA-A3 and HLA-A11 alleles. For detailed KIR ligand status and KIR expression data see Supplemental Tables S1 and S2. * monoallelic data as the NALM-16 cell line has a near-haploid genome. ** Note, that in contrast to theoretical assumptions, this donor does not express KIR3DL1 on the NK cell surface and does therefore lack a $KIR3DL1^{+}$ alloreactive NK cell subset. *** Note, that the assumption of KIR3DL1 is based on genomic data as huNSG mice lacked inhibitory KIR surface expression. **** Genomic expression of KIR2DL2 is absent in this donor. N/A = not applicable.

Donor	KIRL	Donor education of inhibitory KIRs		Educated KIRs specific for the missing KIRL in the target			
		Educated	Uneducated	P3B *Bw6/Bw6 C1/C1*	P23T *ABw4/ABw4 Bw4/Bw4 C2/C1*	P31G *A3/A3 Bw4/Bw6 C2/C2*	Nalm-16 *Bw4 C1**
SNK9A	**Bw6/Bw6 C1/C1**	2DL2, 2DL3	2DL1, 3DL1, 3DL2	/	N/A	2DL2, 2DL3	/
SNK10P	**Bw6/Bw6 C1/C1**	2DL2, 2DL3	2DL1, 3DL1, 3DL2	/	N/A	2DL2, 2DL3	/
SNK21BC	**A3 Bw6/Bw6 C1/C1**	2DL2, 2DL3, 3DL2	2DL1, 3DL1	3DL2	N/A	2DL2, 2DL3	3DL2
SNK13B	**ABw4 Bw4/Bw4 C2/C2**	2DL1, **	2DL2, 2DL3, 3DL2	2DL1, **	N/A	/	2DL1
SNK14B	**ABw4 Bw4/Bw4 C2/C2**	2DL1, 3DL1	2DL2, 2DL3, 3DL2	2DL1, 3DL1	N/A	/	2DL1
SNK15B	**Bw4/Bw4 C2/C2**	2DL1, 3DL1	2DL2, 2DL3, 3DL2	2DL1, 3DL1	N/A	/	2DL1
SNK20B	**A3 Bw4/Bw4 C2/C2**	2DL1, 3DL1, 3DL2	2DL2, 2DL3	2DL1, 3DL1, 3DL2	N/A	/	2DL1, 3DL2
SSC21D	**Bw4/Bw4 C1/C2**	2DL1, 2DL3, 3DL1***, ****	3DL2	2DL1, 3DL1	/	N/A	N/A
SSC18U	**ABw4 Bw4/Bw4 C2/C1**	2DL1, 2DL3, 3DL1, ***	3DL2	2DL1, 3DL1	N/A	N/A	N/A

FIGURE LEGENDS

Figure 1. KIR-KIRL mismatch constellations promote the alloreactivity of cytokine-matured NK cells towards pediatric BCP-ALL *in vitro* and *in vivo*.

(A) *In vitro* NK cell alloreactivity towards BCP-ALL is donor-dependent. Shown is the specific lysis of KIR-KIRL mismatched or matched donors towards the two chosen BCP-ALL samples P3B relapse and P31G (E:T ratio of 10:1). Cytotoxicity towards K562 cells is included as positive control. Data represent two independent experiments performed in triplicates. Donor/patient specific KIR-KIRL repertoire constellations of the 6 donors SNK9A, SNK10P, SNK21BC, SNK13-15B are depicted in the Table 1. (B, C) Donor selection influences the *in vivo* alloreactivity of NKAES cells towards pediatric BCP-ALL. (B) Experimental setup for Figure 1C. (C) Adoptively transferred NKAES cells of a KIR-KIRL mismatched donor (SNK13B) exert higher *in vivo* alloreactivity towards P3B than control NKAES cells of a KIR-KIRL matched donor (SNK10P). Data is representative of one experiment performed with 11 mice.

Figure 2. The KIR^+ NK cell subset of KIR-KIRL mismatched donors exerts higher cytotoxicity towards pediatric BCP-ALL than the corresponding KIR^- subset.

(A) Sorted KIR^+ and KIR^- NK cells of the 7 donors characterized in Table 1 were co-cultured with NALM-16 or K562 cells as a control to determine the extent of *in vitro* cytotoxicity (E:T ratio 5:1). (B) Standardization of the data depicted in Figure 2A. Shown is the specific lysis of the KIR^+ NK cell subset minus the one of the corresponding KIR^- NK cell subset. Data represent six independent experiments with 7 donors performed in triplicates. (C) Interactions of inhibitory KIRs with their cognate ligands determine the extent of NK alloreactivity towards pediatric BCP-ALL. Alloreactivity of sorted KIR^+ NKAES cells of the donors SNK14B, SNK15B, SNK20B (KIR-KIRL mismatch) and SNK10P, SNK21BC (KIR-KIRL match) against NALM16 was determined by *in vitro* killing assays (E:T ratio 2:1) in the

presence or absence of the common inhibitory KIR-blocking mAb IPH2102. Data represent five independent experiments performed in triplicates.

Figure 3. KIR-KIRL mismatch constellations boost the capacity for degranulation and cytokine synthesis of KIR+ NK cells towards pediatric BCP-ALL.

Sorted KIR^+ and KIR^- NK cells of the donors SNK13-15B, SNK20B (donor group "mismatch") were co-cultured with NALM-16 or K562 cells as a control to determine the functional response in terms of degranulation and cytokine synthesis. Pooled data of the intracellular staining showing mean ± SEM. Given are the percentages of the respective $CD107a^+$, $Perforin^+$, TNF^+ and IFN-γ^+ NK cell subpopulation normalized to the corresponding baseline levels of NKAES cells cultured in control medium only. Note, that due to the genuinely higher response the y-axis is differently scaled in K562 experiments. The negative bars indicate the decline in perforin levels which accompanies NK cell degranulation.

Figure 4. The alloreactive KIR^+ NK cell subset of KIR-KIRL mismatched donors exhibits a superior ability for degranulation in response to pediatric BCP-ALL.

(A-D) NKAES cells of KIR-KIRL mismatched (SNK14B, SNK15B, SNK20B) or matched donors (SNK9A, SNK10P, SNK21BC), respectively, were co-cultured with NALM-16 or K562 cells. Shown is the % $CD107a^+$ $CD56^+$ population in various alloreactive or non-alloreactive NK cell subsets. (A) Exemplified gating strategy for the identification of NK cells subsets in donor SNK15B. In relation to NALM-16 cells (Bw4/C1), the potentially alloreactive NK cell population (upper left quadrant) is here represented by the cells expressing KIR2DL1/S1/S4 and/or KIR3DS1 (y-axis) but not KIR2DL2/L3/S2 or KIR3DL1 (x-axis). (Note, that the combined staining with a KIR3DL1/S1 (Z27.3.7) and a KIR3DL1 (DX9) antibody enables the identification of the $KIR3DL1^-S1^+$ cell population that belongs to the alloreactive subset). (B, C) Representative original histogram data obtained from a KIR-KIRL mismatched (SNK15B) and a matched donor (SNK10P). Data in (B) shows CD107a staining in NKAES-only cultures (grey, open) and NKAES cells co-cultured with NALM-16 cells (black, open). Data in (C)

shows the respective stainings in co-culture experiments with K562 (black, open). The upper right pictogram in Figure 4C delineates the respective isotype control (light grey, filled) that allows the subsequent marker position and identification of the $CD107a^+$ subset in B and C. In each histogram the subtraction result ΔCD107a is given (% $CD107a^+$ cells in NKAES-tumor co-cultures minus % $CD107a^+$ cells in NKAES-only cultures). Note, that all experiments were performed with highly activated NKAES cells that exhibit a significant baseline CD107a expression. (D) % $CD107a^+$ $CD56^+$ cells in the indicated NK cells subsets of KIR-KIRL mismatched (SNK14B, SNK15B and SNK20B) or matched donors (SNK9A, SNK10P and SNK21BC). Shown is the increase in CD107a expression of NALM-16 co-cultured KIR^+ NK cell subsets normalized to the CD107a expression of the corresponding KIR^- NK cell subset. Symbols: KIR^+ alloreactive subset in KIR-KIRL mismatched donors: ●, KIR^+ alloreactive subset in matched donors: ○, KIR^+ non-alloreactive NK cell subset: ■. Data represents one experiment performed with the 6 indicated donors.

Figure 5. Immature, KIR^- NK cells exert alloreactivity towards pediatric BCP-ALL *in vivo*.

(A) Experimental setup for humanization of NSG mice. (B) huNSG mice exhibit alloreactivity towards pediatric BCP-ALL *in vivo*. P23T was injected into huNSG or non-humanized control mice and leukemic burden was quantified 20hrs later in the BM. Given is the number of vital blasts normalized to vital murine $CD45^+$ cells. Figure represents pooled data of two independent experiments obtained on a total of 7 huNSG and 3 control mice. (C) Experimental setup for Figure 5D and E. (D) KIR Expression (KIR2DL1/S1/S4, KIR2DL2/L3/S2, KIR3DL1) on BM-derived $CD56^+$ NK cells of huNSG mice. (E) HuNSG-derived NK cells from SSC21D exert significantly higher *in vivo* alloreactivity against P3B than towards P23T. Given is the number of BM-residing vital blasts normalized to vital murine $CD45^+$. Data is representative of two independent experiments with a total of 4 huNSG mice.

Figure 6. "Bridging" therapy with 5-Aza-cytidine supports NK cell alloreactivity towards pediatric BCP-ALL in the early post transplantation period.

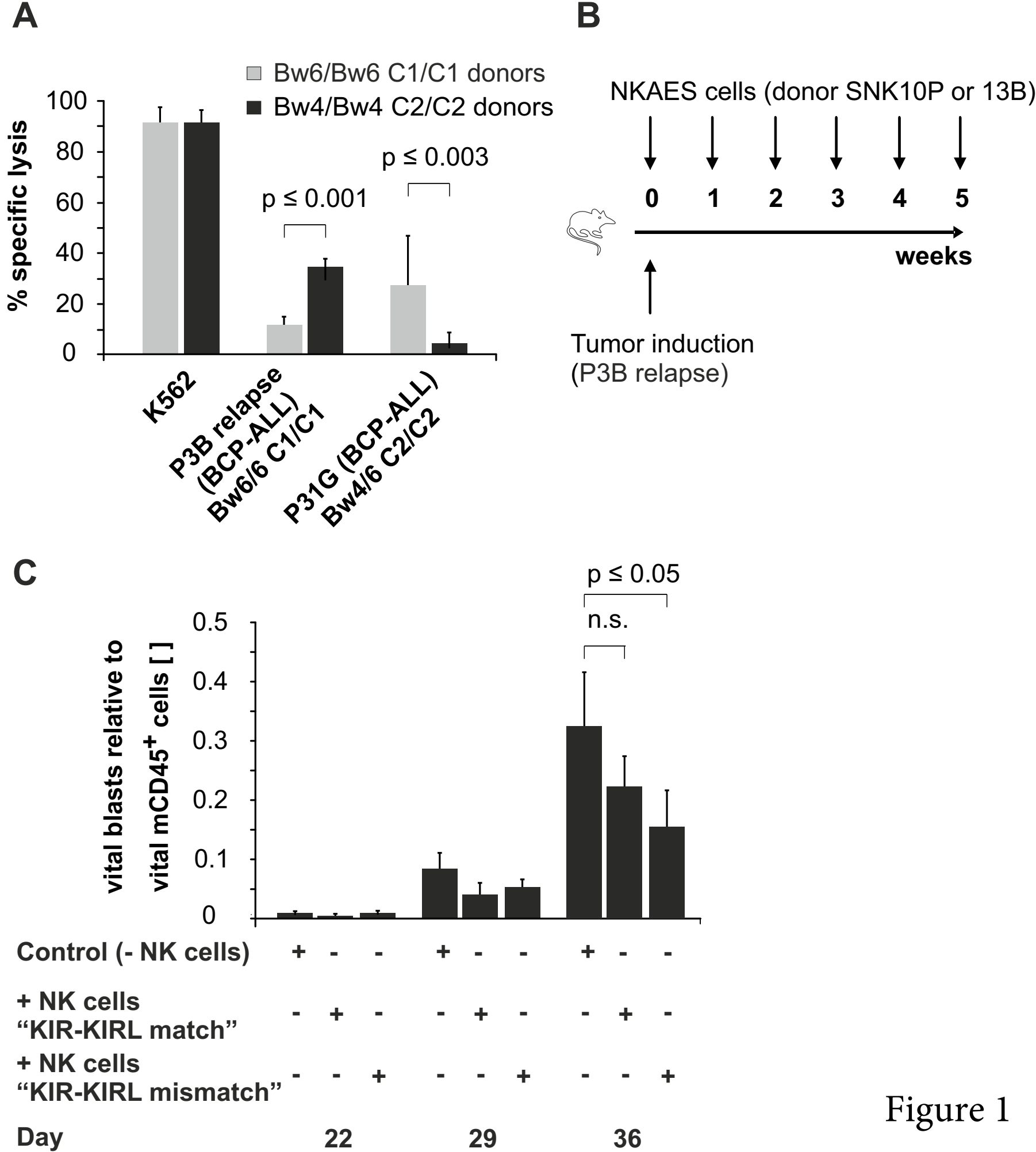
(A) Experimental setup. (B, C) Low-dose and long-term 5-Aza-cytidine treatment does not exert statistically relevant BM cytotoxicity. Given is the number of human (B) or murine (C) $CD45^+$ cells normalized to total live cells. Data depicted in Figure 6B were obtained in 5-Aza-cytidine-treated huNSG mice, data depicted in Figure 6C were obtained in 5-Aza-cytidine-treated non-humanized control mice. (D) Treatment with 5-Aza-cytidine significantly reduces BCP-ALL tumor load in huNSG mice. (E) Low-dose 5-Aza-cytidine treatment regimen does not exert relevant direct cytotoxic effects on pediatric BCP-ALL. Given is the number of vital blasts in the BM normalized to murine $CD45^+$ cells in non-humanized control NSG mice. (F) Calculated effect size of *in vivo* 5-Aza-cytidine treatment on pediatric BCP-ALL burden. "Exp. I and II" denote the two different experiments in huNSG mice, "control I and II" denote the effect in the respective control groups. Data is representative of two independent experiments with a total of 11 huNSG mice and 14 control NSG mice.

Figure 7. Low-dose and long-term exposure to 5-Aza-cytidine promotes NK cell ontogeny.

(A, B) 5-Aza-cytidine does not significantly alter the expression of KIR2DL1/S1/S4, KIR2DL2/L3/S2 and KIR3DL1 (A) or other inhibitory or activating NK cell receptors (B) on BM-derived NK cells of huNSG mice. (C) 5-Aza-cytidine induces the expression of BM-residing NK cell precursors. Frequencies of the indicated NK cell subpopulations in the BM of 5-Aza-cytidine-treated or control huNSG mice (as defined by Freud[23]): preNK: $CD34^+CD117^+$, iNK: $CD34^-CD117^{low}CD94^-$, $CD94^+$ mNK cells: $CD34^-CD117^-CD94^+NKp46^+$, $CD94^-$ mNK cells: $CD34^-CD117^-CD94^-NKp46^+$. Data represent two independent experiments with a total of 11 huNSG mice and 14 control NSG mice.

Figure 8. Chaperoning the NK cell-deficient phase of haplo-identical hematopoietic stem cell transplantation.

Delineation of a hypothetical study design in relapsing pediatric BCP-ALL patients ideally exploits NK cell-mediated anti-tumor responses.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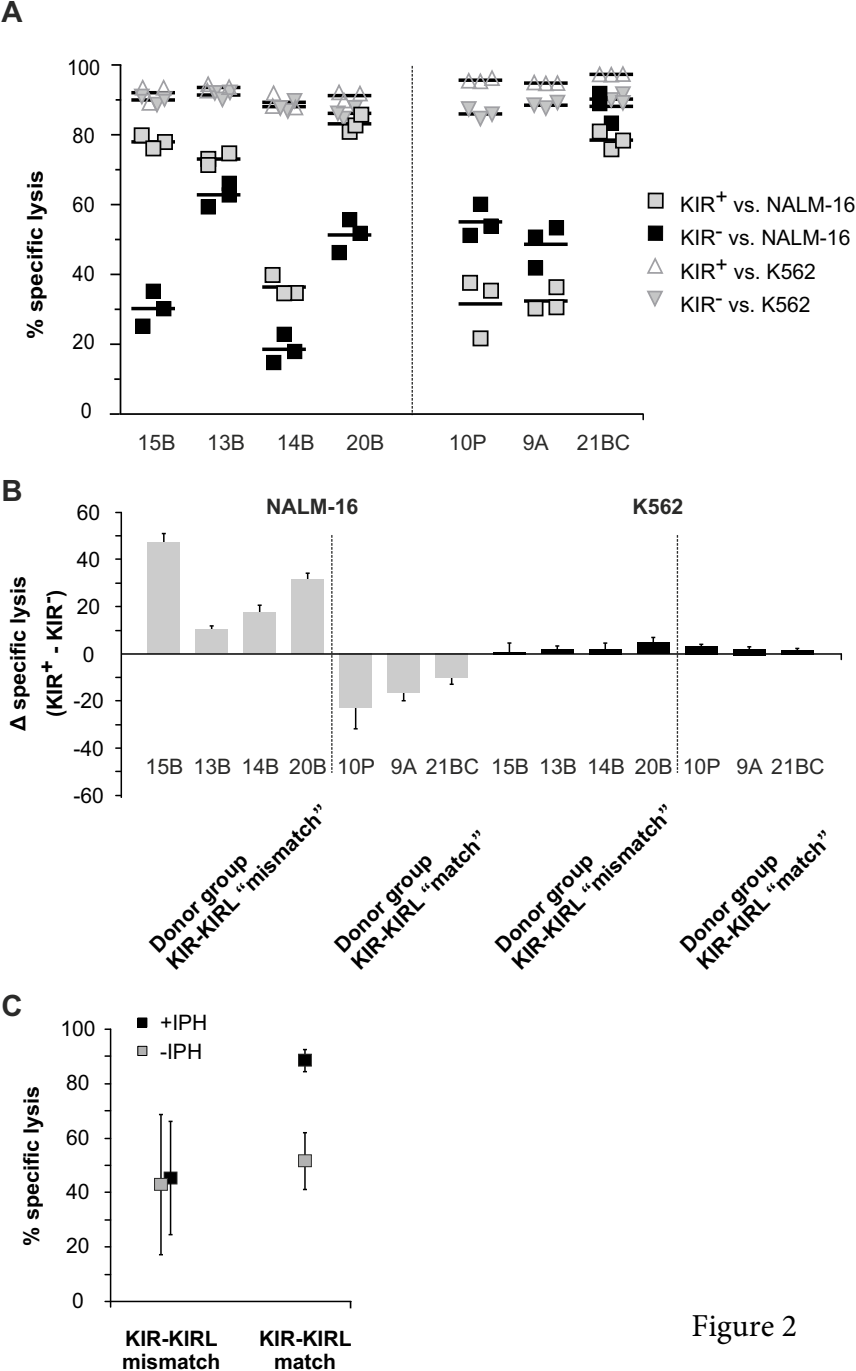
Bw6/Bw6 C1/C1 donors
Bw4/Bw4 C2/C2 donors
% specific lysis
100
80
60
40
20
0
p ≤ 0.001
p ≤ 0.003
K562
P3B relapse (BCP-ALL) Bw6/6 C1/C1
P31G (BCP-ALL) Bw4/6 C2/C2
B
NKAES cells (donor SNK10P or 13B)
0
1
2
3
4
5
weeks
Tumor induction (P3B relapse)
C
p ≤ 0.05
n.s.
vital blasts relative to vital mCD45+ cells [ ]
0.5
0.4
0.3
0.2
0.1
0
Control (- NK cells)
+ NK cells "KIR-KIRL match"
+ NK cells "KIR-KIRL mismatch"
Day
22
29
36

Figure 1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Donor group
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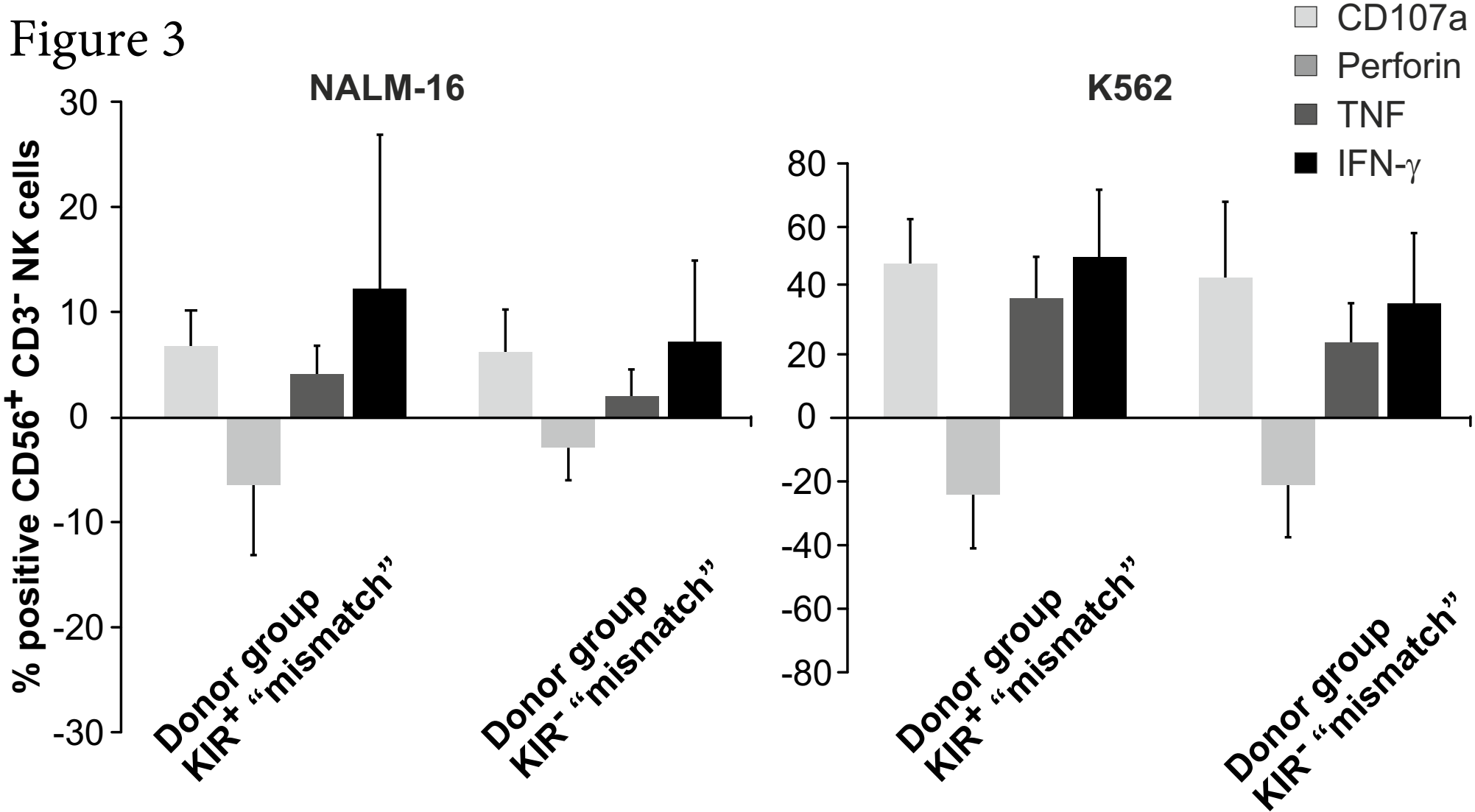
Figure 2

Figure 3


NALM-16
K562
CD107a
Perforin
TNF
IFN-γ
% positive CD56+ CD3- NK cells
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-30
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-40
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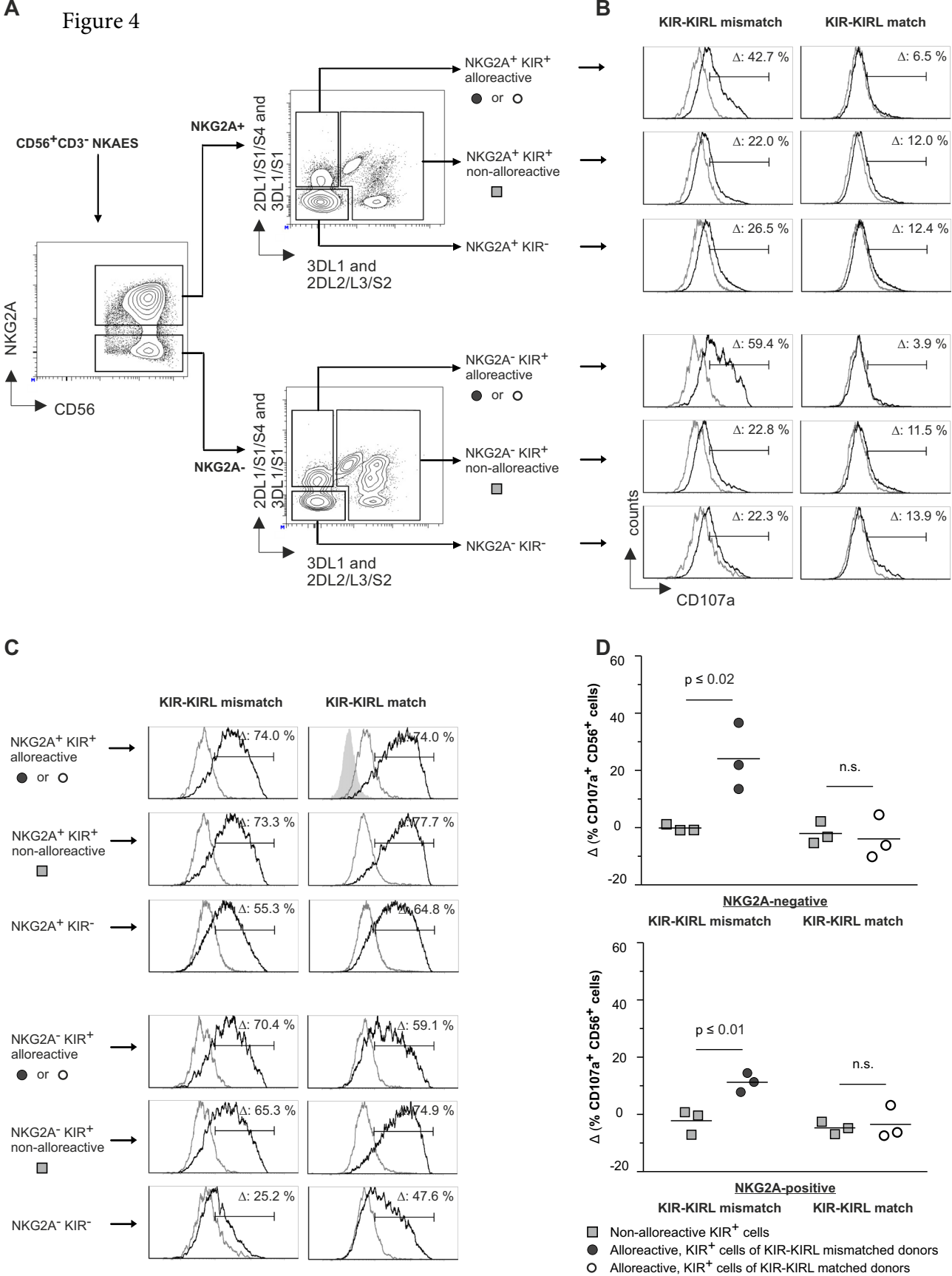
-80
Donor group KIR+ "mismatch"
Donor group KIR- "mismatch"
Donor group KIR+ "mismatch"
Donor group KIR- "mismatch"

Figure 4


A
B
C
D
CD56+CD3- NKAES
NKG2A+
NKG2A-
NKG2A
CD56
2DL1/S1/S4 and 3DL1/S1
3DL1 and 2DL2/L3/S2
NKG2A+ KIR+ alloreactive
or
NKG2A+ KIR+ non-alloreactive
NKG2A+ KIR-
NKG2A- KIR+ alloreactive
NKG2A- KIR+ non-alloreactive
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KIR-KIRL mismatch
KIR-KIRL match
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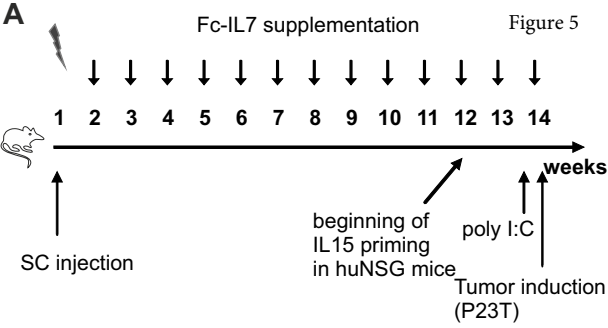
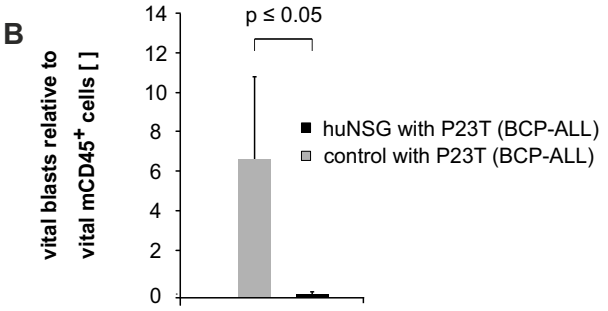
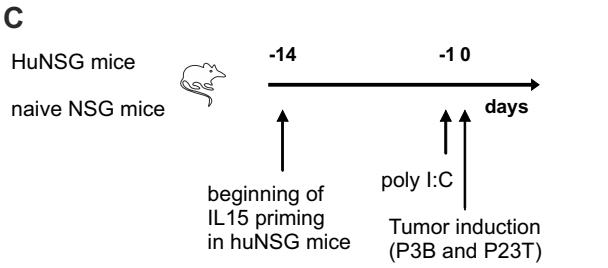
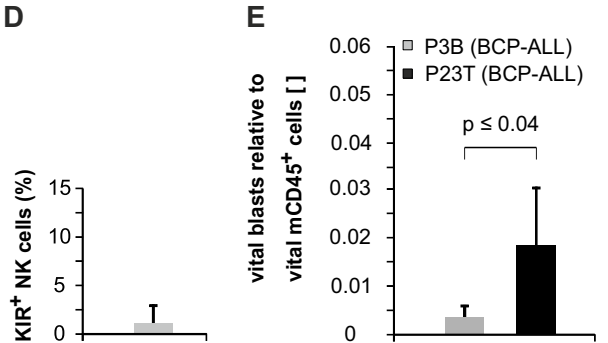
Δ: 6.5 %
Δ: 22.0 %
Δ: 12.0 %
Δ: 26.5 %
Δ: 12.4 %
Δ: 59.4 %
Δ: 3.9 %
Δ: 22.8 %
Δ: 11.5 %
Δ: 22.3 %
Δ: 13.9 %
counts
CD107a
Δ: 74.0 %
Δ: 74.0 %
Δ: 73.3 %
Δ: 77.7 %
Δ: 55.3 %
Δ: 64.8 %
Δ: 70.4 %
Δ: 59.1 %
Δ: 65.3 %
Δ: 74.9 %
Δ: 25.2 %
Δ: 47.6 %
p ≤ 0.02
n.s.
Δ (% CD107a+ CD56+ cells)
NKG2A-negative
p ≤ 0.01
NKG2A-positive
Non-alloreactive KIR+ cells
Alloreactive, KIR+ cells of KIR-KIRL mismatched donors
Alloreactive, KIR+ cells of KIR-KIRL matched donors


A
Fc-IL7 supplementation
1 2 3 4 5 6 7 8 9 10 11 12 13 14
weeks
SC injection
beginning of IL15 priming in huNSG mice
poly I:C
Tumor induction (P23T)


B
vital blasts relative to vital mCD45+ cells [ ]
p ≤ 0.05
huNSG with P23T (BCP-ALL)
control with P23T (BCP-ALL)
0 2 4 6 8 10 12 14


C
HuNSG mice
naive NSG mice
-14
-1 0
days
beginning of IL15 priming in huNSG mice
poly I:C
Tumor induction (P3B and P23T)


D
KIR+ NK cells (%)
0 5 10 15
E
vital blasts relative to vital mCD45+ cells [ ]
P3B (BCP-ALL)
P23T (BCP-ALL)
p ≤ 0.04
0 0.01 0.02 0.03 0.04 0.05 0.06

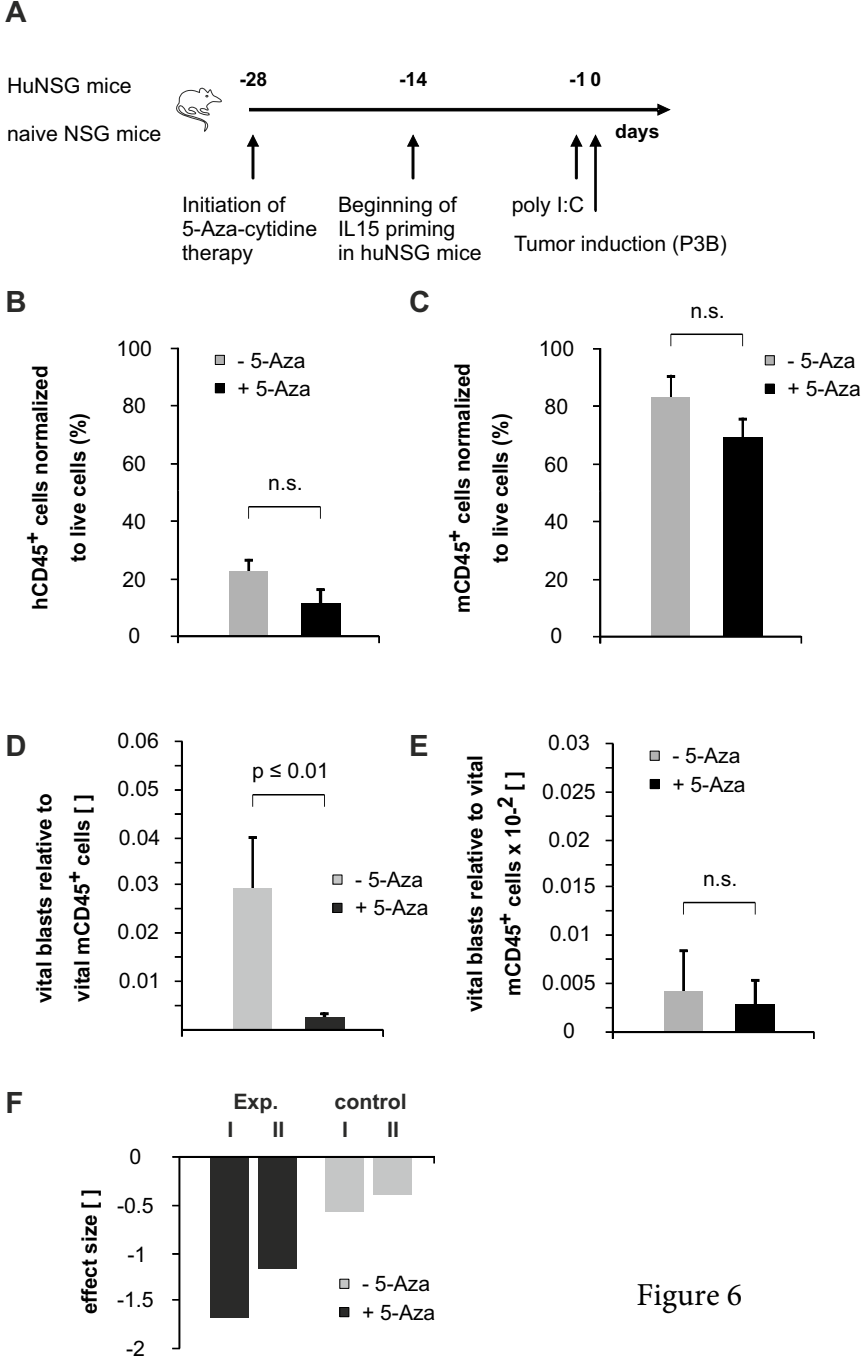

A
HuNSG mice
naive NSG mice
-28
-14
-10
days
Initiation of 5-Aza-cytidine therapy
Beginning of IL15 priming in huNSG mice
poly I:C
Tumor induction (P3B)
B
hCD45+ cells normalized to live cells (%)
- 5-Aza
+ 5-Aza
n.s.
C
mCD45+ cells normalized to live cells (%)
n.s.
D
vital blasts relative to vital mCD45+ cells [ ]
p ≤ 0.01
E
vital blasts relative to vital mCD45+ cells x 10-2 [ ]
n.s.
F
Exp.
control
I
II
effect size [ ]

Figure 6

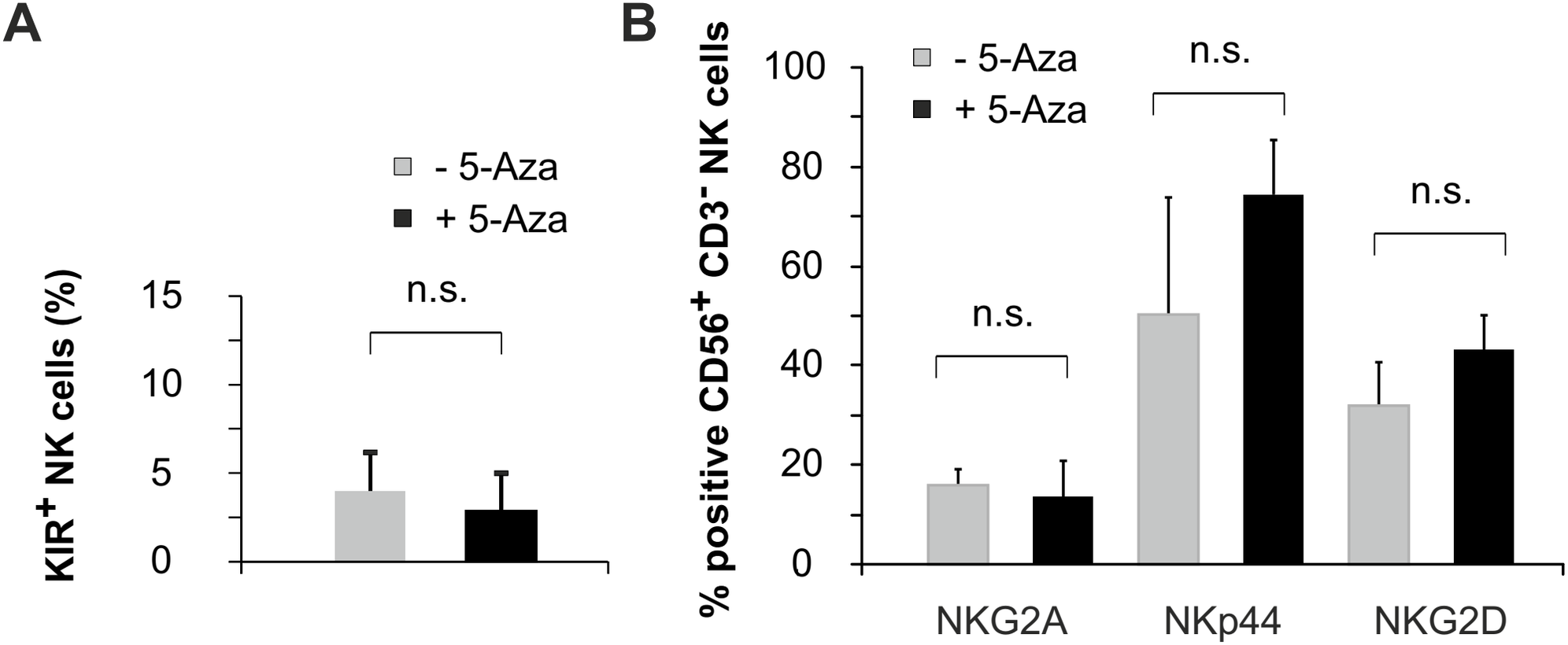

A
- 5-Aza
+ 5-Aza
KIR+ NK cells (%)
15
10
5
0
n.s.
B
- 5-Aza
+ 5-Aza
% positive CD56+ CD3- NK cells
100
80
60
40
20
0
n.s.
n.s.
n.s.
NKG2A
NKp44
NKG2D

Figure 7

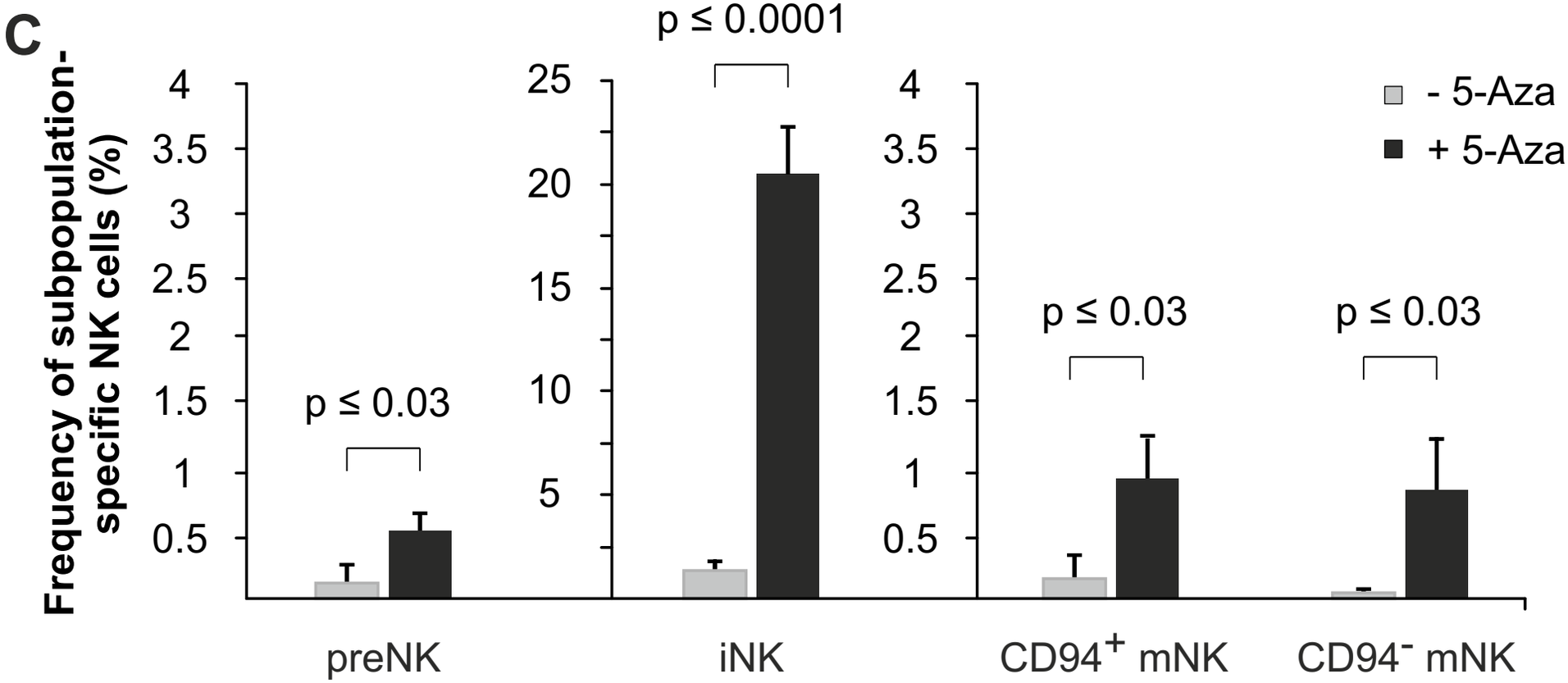
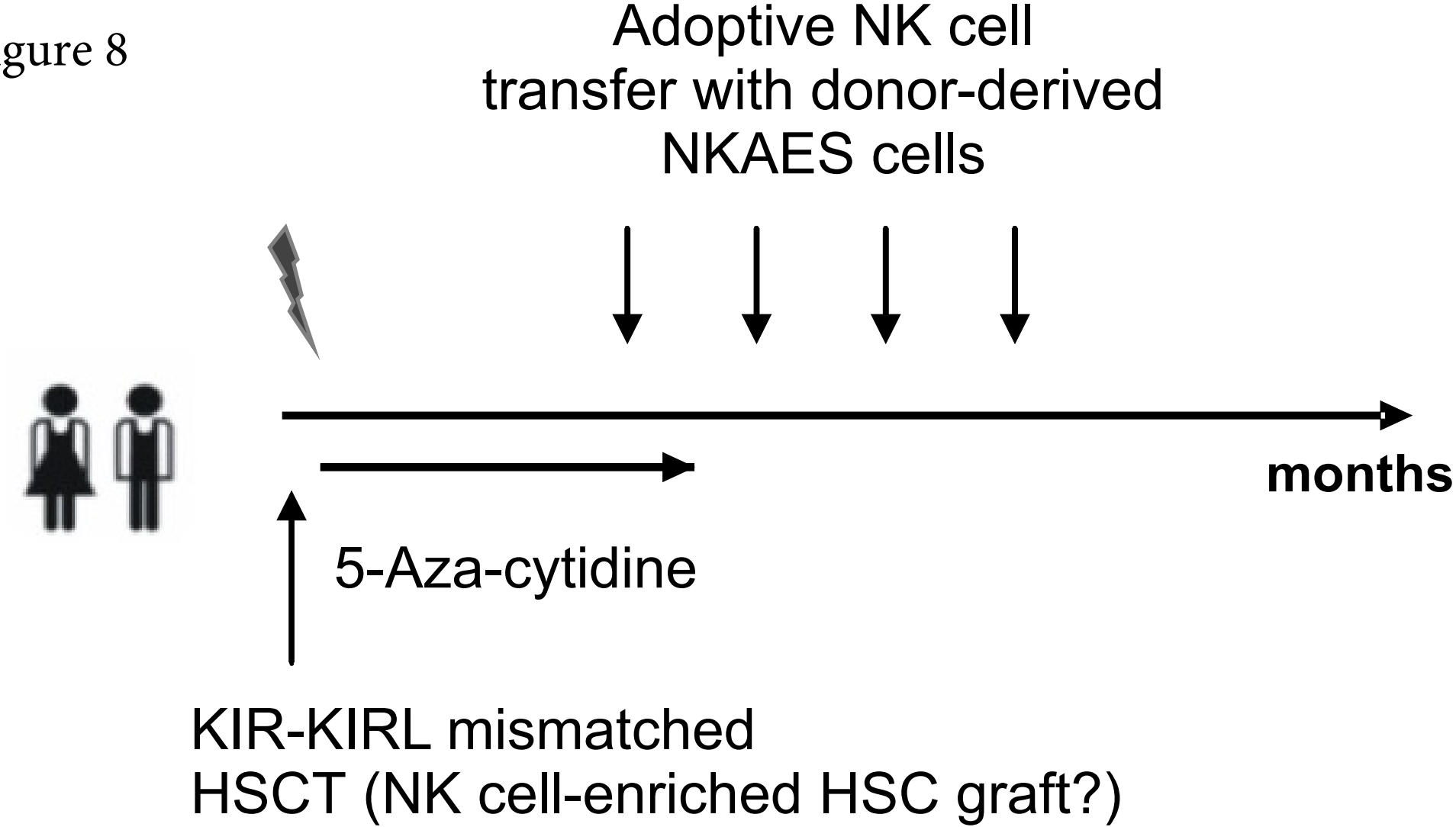

C
Frequency of subpopulation-specific NK cells (%)
- 5-Aza
+ 5-Aza
p ≤ 0.03
p ≤ 0.0001
p ≤ 0.03
p ≤ 0.03
preNK
iNK
CD94+ mNK
CD94- mNK

Figure 8


Adoptive NK cell
transfer with donor-derived
NKAES cells
months
5-Aza-cytidine
KIR-KIRL mismatched
HSCT (NK cell-enriched HSC graft?)

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Both mature KIR^{+} and immature KIR^{-} NK cells control pediatric acute B cell precursor leukemia in NOD.Cg-Prkdc ^{scid} IL2rg^{tmWjl}/Sz mice

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